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(54) Title: PLASTID TRANSFORMATION OF *BRASSICA*

(57) Abstract: A method is provided for transforming *Brassica* plants to express DNA sequences of interest from the plant cell plastid. The method allows the transformation of *Brassica* plant tissue with heterologous DNA constructs. Such DNA constructs comprise, in the 5' to 3' direction of transcription, a promoter region functional in a plant plastid and a DNA sequence of interest. The invention further provides for *Brassica* cells in which the plastids contain heterologous DNA constructs.

PLASTID TRANSFORMATION OF *BRASSICA*

INTRODUCTION

Technical Field

The invention relates to methods of genetically transforming plant plastids, and more specifically to genetically transforming the plastid genomes of *Brassica* plant species.

Background

The plastids of higher plants are an attractive target for genetic engineering. Plant plastids (chloroplasts, amyloplasts, elaioplasts, etioplasts, chromoplasts, etc.) are the major biosynthetic centers that, in addition to photosynthesis, are responsible for production of industrially important compounds such as amino acids, complex carbohydrates, fatty acids, and pigments. Plastids are derived from a common precursor known as a proplastid and thus the plastids present in a given plant species all have the same genetic content. In general, plant cells contain 500-10,000 copies of a small 120-160 kilobase circular genome, each molecule of which has a large (approximately 25kb) inverted repeat. Thus, it is possible to engineer plant cells to contain up to 20,000 copies of a particular gene of interest which potentially can result in very high levels of foreign gene expression. In addition, plastids of most plants are maternally inherited. Consequently, unlike heterologous genes expressed in the nucleus, heterologous genes expressed in plastids are not pollen disseminated, therefore, a trait introduced into a plant plastid will not be transmitted to wild-type relatives.

Plastids of higher plants present an attractive target for genetic engineering. As mentioned above, plastids of higher plants are maternally inherited. This offers an advantage for genetic engineering of plants for tolerance or resistance to natural or chemical conditions, such as herbicide tolerance, as these traits will not be transmitted to wild-type relatives. A review of plastid transformation of flowering plants is provided by Maliga (1993) *Trends in Biotech.* 11:101-107, the entirety of which is incorporated herein by reference.

Unfortunately, successful plastid transformation techniques described thusfar for higher plants have been limited to model crop plants such as tobacco (U.S. Patent Number

5,451,513; Svab *et.al.* (1990), *Proc. Natl. Acad. Sci. USA* 87:8526-8530 and Svab *et al.* (1993), *Proc. Natl. Acad. Sci. USA* 90:913-197) and *Arabidopsis* (Sikdar, *et al.* (1998) *Plant Cell Reports* 18:20-24). Furthermore, the methods described for *Arabidopsis* plants, produce infertile regenerates. PCT Publication WO 97/32977 also describes methods for the plastid transformation of *Arabidopsis* and provides prophetic examples of plastid transformation of *Brassica* plastids. However, transplastomic *Brassica* plants have not been produced to date using the methods described therein. Thus, for practical applications of genetic engineering techniques to crop plant plastids, chloroplast transformation techniques for a wide variety of crop plants, such as *Brassica* species, are needed in the art.

Chloroplast transformation methods applicable to crop species, such as *Brassica* species, are needed in the art. Such methods would provide for a novel means of genetic engineering via plastid transformation for agronomically as well as qualitatively important traits via genetic engineering of plant plastids.

SUMMARY OF THE INVENTION

The present invention provides constructs and methods for the transformation and regeneration of *Brassica* plants containing plant cells, the plastids of which have been stably transformed by a foreign DNA of interest. The method generally comprises transforming a *Brassica* plant cell plastid with a DNA construct having regions of homology obtained from the *Brassica* plastid genome; selecting for cells which contain the DNA construct; and obtaining a mature multicellular plant, the cells of which contain the DNA construct in the plant cell plastid.

A first aspect of the present invention provides methods for transforming the plastids of *Brassica* plant cells with a DNA construct generally comprising, in the 5' to 3' direction of transcription, a promoter region functional in a plant cell plastid, a DNA sequence of interest, and a transcription termination region functional in a plant cell plastid. The constructs further comprise regions of homology derived from *Brassica* plastid sequences.

Another aspect of the present invention provides nucleic acid constructs having a promoter functional in a *Brassica* plant cell plastid, a nucleic acid sequence of interest, and a transcription termination sequence functional in a plant cell plastid. The constructs can also contain regions of homology for the integration of the construct into the host cell plastid

genome. The regions of homology are preferably obtained from the *Brassica* plastid genome sequence.

In another aspect of the present invention, methods for expressing a nucleic acid sequence in a host *Brassica* plant cell plastid are provided. In general, the methods involve introducing into a host *Brassica* plant cell a recombinant nucleic acid construct having a promoter functional in a plastid, a nucleic acid sequence of interest, and a transcriptional termination sequence functional in a plant cell plastid.

In yet another aspect of the present invention, multicellular *Brassica* plant obtained by the methods described herein are provided.

The invention also provides a multicellular *Brassica* plant, the plastids of which have been transformed with a DNA construct of interest.

The invention also provides a method for obtaining a *Brassica* plant cell, of which the plastid has been stably transformed with a DNA construct, comprising in the 5' to 3' direction of transcription, a promoter functional in a plant cell plastid, a DNA sequence encoding a green fluorescent protein (herein referred to as GFP), and a transcriptional termination region functional in a plant cell plastid.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a construct for preparation of transplastomic *Brassica* lines containing a GFP expression marker. A schematic of the pCGN6408 construct and representation of incorporation into the tobacco plastid genome are shown at the top. Lines above the *aad*A and GFP boxes indicate the direction of transcription. Expected sizes for *Stu*I fragments are provided for the incoming DNA as well as the wild type DNA (ptDNA).

Figure 2 provides the results of Southern hybridizations for nontransformed (wild-type, Bn) and transplastomic *Brassica* lines.

Figure 3 provides Southern Hybridization results for subsequent rounds of shoots, lines 6408 99-1Y, cc, and dd are about 80% homoplasmic.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, constructs and methods are provided for obtaining *Brassica* plant cells containing plastids into which heterologous DNA has been

inserted. The method generally encompasses transforming a *Brassica* plant cell with plastid expression vectors. The plastid expression constructs generally contain nucleic acid sequences comprising, as operably associated components in the 5' to 3' direction of transcription, a promoter region functional in a plant plastid, a DNA sequence of interest, and a transcription termination region capable of terminating transcription in a plant plastid. The constructs for use preferably comprise regions of homology for integration into the host plant cell plastid genome. The regions of homology are preferably obtained from *Brassica* genome sequence. The term "obtained" as used herein refers to any sequence analogous to a region of homology of a *Brassica* plastid genome sequence. Such sequence can be produced in accordance with any method known in the art and include but not limited to synthesis, amplification from a plastid genomic sequence, or cloned from *Brassica* plastid genomic sequences.

In one aspect of the present invention a method of producing a plant cell from a *Brassica* plant species wherein the plastids of the plant contain a recombinant nucleic acid construct to direct the expression of a nucleic acid sequence of interest from the plant cell plastid.

The methods generally comprise introducing recombinant nucleic acid constructs into plant cell plastids in prepared tissue sources from *Brassica* plant species. The nucleic acid constructs can be integrated into the plastid genome, or can be contained as self-replicating plasmids within the plastid. Preferably, the constructs are integrated into the plant cell plastid genome using homologous recombination sequences obtained from a *Brassica* plastid genome.

The plant cell used in the methods of the present invention can be obtained from any plant tissue source which contain plastids, and which has the ability to regenerate into a mature plant or structure which will give rise to a mature plant. Such tissues include, but are not limited to, leaf tissue, cotyledons (including cotyledonary notch), hypocotyls, epicotyls, stem sections, embryogenic callus, callus, petioles, protoplasts, stem thin layers, microspores, as well as some seeds and embryos. Preferably, the cells used in the methods of the present invention are obtained from leaf tissue. Furthermore, the tissue source can be derived from plants grown in a variety of conditions, including *in vitro*, soil grown, and the like.

Prior to transformation, the tissue source can be treated with various osmotic pressures. Osmotic treatment refers to culturing the tissue on a medium containing sugar alcohols, including but not limited to sorbitol and mannitol. The osmotic treatment can also

be performed after transformation, during transformation, or before, during and after transformation. Concentrations of sugar alcohols for use in the treatments include ranges from about 0.01M to about 1.0M, preferably from about 0.05M to about 0.7M, more preferably from about 0.1M to about 0.5M, most preferably from about 0.2M to about 0.4M.

Constructs for transformation into a Brassica plant cell can be introduced using any available method. Methods for introduction include, but are not limited to, biolistic transformation, PEG mediated transformation, and electroporation. Preferably, DNA constructs of interest are transformed into the plastids of a plant cell from a desirable host tissue using particle gun bombardment. General methods for biolistic transformations are described by Sanford, *et al.* (1993) *Methods in Enzymology* 217:483-509, and Ye, *et al.* (1990) *Plant Molecular Biology* 15:809-819. Stable transformation of tobacco plastid genomes by particle bombardment is reported (Svab *et.al.* (1990 *supra*) and Svab *et al.* (1993 *supra*)). The methods reported therein, can be employed in the transformation methods of the present invention. Other methods are known in the art, and are described by O'Neil, *et al.* (1993) *Plant Journal* 3:729-738 and Golds, *et al.* (1993) *Bio/Technology* 11:95-97.

The regeneration of whole plants from a transformed cell contained in the transformed tissue used in transformation involves several growth stages. Typically, a tissue, having been excised from an adult plant or germinated seedling, is placed in a chemically defined medium under sterile conditions. By growing the explant under such controlled conditions for a period of time, an undifferentiated mass of cells, referred to as a callus, can form.

By culturing this callus under the proper set of conditions, e.g., nutrients, light, temperature, humidity, and by providing the proper combination and concentration of plant growth regulators, the calli can be induced to form differentiated cells and regenerate plant shoots. Plant shoots, sometimes referred to as plantlets, containing meristem tissue are then transferred to a media for the induction of root production.

Preferably, tissue into which a plastid construct has been introduced is cultured on a cell division, or cell expansion, promoting media, referred to as delay medium. The delay medium can be either liquid or solid, or semi-solid by the addition of a solidifying agent, such as agar. The tissue is preferably cultured on delay medium for a period ranging from about 0 days to about 14 days, preferably from about 1 day to about 10 days, more preferably from about 1 day to about 7 days, most preferably from about 2 days to about 7 days, after which the plant tissue is transferred to a selective media containing an inhibitory amount of the particular selective agent, as well as the particular hormones and other substances necessary

to obtain regeneration for that particular plant species. Shoots are then subcultured on the same selective media, containing higher, lower or the same concentration of the particular selective agent, to ensure production and selection of homoplasmic shoots.

The selective media can be liquid or solid or semi-solid by the addition of a solidifying agent, such as agar. Liquid selective media allows for greater surface area of contact of the plant tissue with the selective media containing particular hormones, particular selective agent and other substances necessary to obtain regeneration.

The amount of selective agent can remain constant in the media during regeneration. Alternatively, the amount of selective agent can initially be at higher levels, then lowered during later stages of regeneration. Furthermore, the selective agent amount can be lower during the initial stages of regeneration, then increased later in regeneration.

Transplastomic plants are analyzed for a pure population of transformed plastid genomes (homoplasmic lines). Homoplasmy can be verified using Southern analysis employing nucleic acid probes spanning a region of the transgene and chloroplast genome (i.e. the insertion region). Transplastomic plants which are heteroplasmic (i.e. contain a mixture of plastid genomes containing and lacking the transgene) are characterized by a hybridization pattern of wild type and transgenic bands. Homoplasmic plants show a hybridization pattern lacking the wild type band.

Alternatively, homoplasmy can be verified using the polymerase chain reaction (PCR). PCR primers are utilized which are targeted to amplify from sequences from the insertion region. For example, a pair of primers can be utilized in a PCR reaction. One primer amplifies from a region in the transgene, while the second primer amplifies from a region proximal to the insertion region towards the insertion region. A second PCR reaction is performed using primers designed to amplify the region of insertion. Transplastomic lines identified as homoplasmic produce the expected size fragment in the first reaction, while they do not produce the predicted size fragment in the second reaction.

As described in more detail in the examples below, transplastomic *Brassica* plants are produced from methods described herein.

Other *Brassica* plant species can be similarly transformed using the methods of the present invention. Suitable plants for the practice of the present invention include, but are not limited to, diploid *Brassica* species including *B. rapa*, *B. oleracea*, and *B. nigra*, as well as amphidiploid species including *B. napus*, *B. juncea*, and *B. carinata*. The methods of the

present invention can also find use in the transformation of plastids of closely related plants such as *Arabidopsis*.

Another aspect of the present invention provides recombinant nucleic acid constructs having a promoter functional in a plant cell plastid, a nucleic acid sequence of interest, and a transcriptional termination region functional in a plant cell plastid.

As used herein, a "recombinant nucleic acid construct" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a target cell. The recombinant nucleic acid construct includes an expression cassette which can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of a nucleic acid construct includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter.

A number of promoters are available to the skilled artisan which are functional in plant cell plastids. Such promoters include, but are not limited to, those derived from promoter regions of highly expressed plastid genes such as the promoter regions from the 16S ribosomal RNA operon (P*rrn*), psbA gene (PpsbA) or the rbcL gene (PrbcL). Additional elements can also be employed with the promoter regions to enhance transcription, translation, or both. Such elements include, but are not limited to, ribosome binding sites, Shine-Delgarno sequences, enhancer elements, and the like.

The nucleic acid sequence for use in the recombinant construct can be any nucleic acid sequence of interest for transcription or transcription and translation (expression) in a host plant cell plastid. Sequences of interest include but are not limited to sequences encoding for genes involved in tolerance to herbicides, reporter genes, selectable markers, mammalian proteins, and pathogen resistance.

Nucleic acid sequences encoding for proteins involved in herbicide tolerance are known in the art, and include, but are not limited to DNA sequences encoding for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, described in U.S. Patent Numbers 5,627,061, and 5,633,435, Padgette *et al.* (1996) *Herbicide Resistant Crops*, Lewis Publishers, 53-85, and in Penaloza-Vazquez, *et al.* (1995) *Plant Cell Reports* 14:482-487) and aroA (U.S. Patent Number 5,094,945) for glyphosate tolerance, bromoxynil nitrilase (Bxn) for Bromoxynil tolerance (U.S. Patent Number 4,810,648), phytoene desaturase (crtI (Misawa et al, (1993) *Plant Journal* 4:833-840, and (1994) *Plant Jour* 6:481-489) for tolerance to norflurazon, acetohydroxyacid synthase (AHAS (Sathasiivan *et al.* (1990) *Nucl.*

Acids Res. 18:2188-2193)) and the *bar* gene for tolerance to glufosinate (DeBlock, *et al.* (1987) *EMBO J.* 6:2513-2519.

It should be noted that the expression constructs of the present invention can also include sequences encoding genes involved in other stress tolerances, for example insect or disease resistance/tolerance genes. Such insect tolerance genes are known in the art, for example the *Bacillus thuringensis cry*1Ac protein.

In addition, the expression constructs also find use in directing the production of human biological proteins (pharmaceutical proteins) from the plant plastid. Nucleic acid sequences encoding for the Human Growth Hormone (hGH) can be employed in the plastid expression constructs of the present invention.

Other sequences of interest for use in the expression constructs of the present invention for the production of human biological proteins is the production of aprotinin.

Other sequences which find use in the production of human biologics include sequences encoding for insulin or insulin precursors. The skilled artisan will recognize that many nucleotide sequences encoding for human biologics can be employed in the constructs of the present invention to direct their expression from a plant plastid such as those described in Goodman and Gelman (1990) *Pharmacological Basis of Therapeutics*, Pergaman Press, 8th Edition, Sections 14 and 15.

A number of reporter genes are available to the skilled artisan, and include but are not limited to GUS and green fluorescent protein (GFP). Analysis techniques for the expression of GUS from transgenic tissues involves destruction of tissues prior to staining. Generally, the tissue is infiltrated with a glucoronide containing solution, then destained with an alcohol solution to remove chlorophyll background. The stained tissue is then visually observed for GUS staining, as evidenced by a blue coloration of the cells expressing b-glucoronidase. Other reporter genes are also known in the art, for example the Green Fluorescent Protein (GFP) can be employed using non-destructive analysis methods. A general review of GFP is provided by Tsien (1998) *Annu. Rev. Biochem.* 67:509-544, the entirety of which is incorporated herein by reference.

As discussed in more detail in the examples that follow, constructs employing GFP are used to transform *Brassica* plants such that the transformed *Brassica* plant has integrated into the chloroplast genome the construct to direct the expression of the GFP from the plastid. Cells of plants expressing GFP can be visualized under ultraviolet (uv) light, without the need for destructive methods. Visualized under uv light, the cells expressing GFP fluoresce as a

green color. Mutations in the GFP coding sequence shift the excitation wavelength to blue light, allowing for a more convenient visualization of expression on a green plant surface.

Furthermore, transplastomic *Brassica* plants are identified which are heteroplasmic for the DNA sequences of interest encoding the GFP gene. Transplastomic *Brassica* plants are obtained using the methods of the present invention to transform a DNA construct comprising a marker gene, such as GFP, expressed from a promoter sequence which is functional in a plant cell plastid. The transplastomic plant obtained herein demonstrate GFP expression as determined by visualization under uv microscopy.

In developing the constructs the various fragments comprising the regulatory regions and open reading frame can be subjected to different processing conditions, such as ligation, restriction enzyme digestion, PCR, *in vitro* mutagenesis, linkers and adapters addition, and the like. Thus, nucleotide transitions, transversions, insertions, deletions, or the like, can be performed on the DNA which is employed in the regulatory regions or the DNA sequences of interest for expression in the plastids. Methods for restriction digests, Klenow blunt end treatments, ligations, and the like are well known to those in the art and are described, for example, by Maniatis *et al.* (in *Molecular cloning: a laboratory manual* (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

During the preparation of the constructs, the various fragments of DNA will often be cloned in an appropriate cloning vector, which allows for amplification of the DNA, modification of the DNA or manipulation of the DNA by joining or removing sequences, linkers, or the like. Preferably, the vectors will be capable of replication to at least a relatively high copy number in *E. coli*. A number of vectors are readily available for cloning, including such vectors as pBR322, vectors of the pUC series, the M13 series vectors, and pBluescript vectors (Stratagene; La Jolla, CA).

In order to provide a means of selecting the desired plant cells, vectors for plastid transformation typically contain a construct which provides for expression of a selectable marker gene. Marker genes are plant-expressible DNA sequences which express a polypeptide which resists a natural inhibition by, attenuates, or inactivates a selective substance, *i.e.*, antibiotic, herbicide *etc*..

Alternatively, a marker gene can provide some other visibly reactive response, *i.e.*, can cause a distinctive appearance or growth pattern relative to plants or plant cells not expressing the selectable marker gene in the presence of some substance, either as applied directly to the plant or plant cells or as present in the plant or plant cell growth media.

In either case, the plants or plant cells containing such selectable marker genes will have a distinctive phenotype for purposes of identification, *i.e.*, they will be distinguishable from non-transformed cells. The characteristic phenotype allows the identification of cells, cell groups, tissues, organs, plant parts or whole plants containing the construct.

Detection of the marker phenotype makes possible the selection of cells having a second gene to which the marker gene has been linked. This second gene typically comprises a desirable phenotype which is not readily identifiable in transformed cells, but which is present when the plant cell or derivative thereof is grown to maturity, even under conditions wherein the selectable marker phenotype itself is not apparent.

The use of such a marker for identification of plant cells containing a plastid construct has been described by Svab *et al.* (1993, *supra).* In the examples provided below, a bacterial *aad*A gene is expressed as the marker under the regulatory control of chloroplast 5' promoter and 3' transcription termination regions, specifically the regulatory regions of the*psb*A gene (described in Staub *et al.*, *EMBO J.*(1993) 12(2):601-606). Numerous additional promoter regions can also be used to drive expression of the selectable marker gene, including various plastid promoters and bacterial promoters which have been shown to function in plant plastids.

Expression of the *aad*A gene confers resistance to spectinomycin and streptomycin, and thus allows for the identification of plant cells expressing this marker. The *aad*A gene product allows for continued growth and greening of cells whose chloroplasts comprise the selectable marker gene product. Cells which do not contain the selectable marker gene product are bleached or purple in color. Selection for the *aad*A gene marker is thus based on identification of plant cells which are not bleached by the presence of streptomycin, or more preferably spectinomycin, in the plant growth medium.

A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. Other genes which encode a product involved in chloroplast metabolism can also be used as selectable markers. For example, genes which provide resistance to plant herbicides such as glyphosate, bromoxynil or imidazolinone can find particular use. Such genes have been reported (Stalker *et al.*, *J. Biol. Chem.* (1985) *260*:4724-4728 (glyphosate resistant EPSP); Stalker *et al.*, *J. Biol. Chem.* (1985) *263*:6310-6314 (bromoxynil resistant nitrilase gene); and Sathasivan *et al.*, *Nucl. Acids Res.* (1990) *18*:2188 (AHAS imidazolinone resistance gene)).

The vectors for use in plastid transformation can include sequences to provide for an origin of replication to allow the introduced construct to replicate autonomously in the plastid. Such sequences are known in the art and are described in U.S. Patent Number 5,693,507, the entirety of which is incorporated herein by reference.

The vectors for use in plastid transformation preferably include means for providing a stable transfer of the plastid expression construct and selectable marker construct into the plastid genome. This is most conveniently provided by regions of homology to the target plastid genome. The regions of homology flank the expression construct to be transferred and provide for transfer to the plastid genome by homologous recombination, via a double crossover into the genome. The complete DNA sequence of the plastid genome of tobacco has been reported (Shinozaki *et al.*, *EMBO J.* (1986) *5*:2043-2049). Complete DNA sequences of the plastid genomes from liverwort (Ohyama *et al.*, *Nature* (1986) *322*:572-574) and rice (Hiratsuka *et al.*, *Mol. Gen. Genet.* (1989) *217*:185-194), have also been reported.

Where the regions of homology are present in the inverted repeat regions of the plastid genome (known as IRA and IRB), two copies of the transgene are expected per transformed plastid. Where the regions of homology are present outside the inverted repeat regions of the plastid genome, one copy of the transgene is expected per transformed plastid. The regions of homology within the plastid genome are approximately 1kb to 3kb in size. Smaller regions of homology can also be used, and as little as 100 bp can provide for homologous recombination into the plastid genome. However, the frequency of recombination and thus the frequency of obtaining plants having transformed plastids decreases with decreasing size of the homology regions.

Examples of constructs having regions of homology the tobacco plastid genome are described in Svab *et.al.* (1990 *supra*), Svab *et al.* (1993 *supra*) and Zoubenko *et al.* (*Nuc Acid Res* (1994) 22(19):3819-3824). Examples of constructs having regions of homology to the *Arabidopsis* plastid genome are described by Sikdar, *et al.* (1998) *supra*.

As described herein, constructs are prepared using regions of homology derived from the sequences of the *Brassica* plastid genome to direct the homologous recombination of the heterologous DNA into the inverted repeat region of the plastid genome. Such regions of homology are obtained by utilizing PCR reactions to isolate sequences corresponding to the regions of homology in *Brassica* (also referred to herein as a homologous plastid genome). Thus, as used herein, regions of homology to a homologous plastid genome refers to DNA sequences which are used in the preparation of constructs to direct the integration of the

expression construct into the plastid genome of the same plant genus as that from which the regions are derived.

The skilled artisan will recognize that additional regions of homology can also be utilized in accordance with the methods of the present invention. For example, regions of homology derived from other plant species plastid genomes can also be employed, for example, from *Arabidopsis*, or tobacco. Also, the regions of homology can also provide for homologous recombination in the plastid genome corresponding to the large single copy region.

Furthermore, the expression constructs provided herein utilize regulatory elements derived from the *Brassica* plastid genome. For example the promoter region obtained from *Brassica* 16S ribosomal RNA (Prrn) is used for the expression of the selectable marker *aad*A. Other regulatory regions derived from the *Brassica* plastid genome are also used in the constructs of the present invention, such as the terminator sequence of rps16 as well as both the promoter and transcription termination regions of *psb*A. The skilled artisan will recognize that regulatory elements derived from heterologous plastid genomes, such as Tobacco or *Arabidopsis* can also be used in the constructs of the present invention.

Also contemplated in the present invention is the introduction of two or more constructs into a single *Brassica* plant cell plastid. Such methods include, but are not limited to retransformation, co-transformation, and the like. Methods for co-transformation of plastids have been described for tobacco by Carrer, *et al.* (1995) *Bio/technology*, 13:791-794. Such methods allow for selection on one selective agent, followed by selection on a second selective agent. For example, one construct could contain an aadA expression cassette for selection on spectinomycin/streptomycin, and a second construct could contain a CP4 EPSPS expression cassette for selection on glyphosate.

Thus, expression constructs for use in the methods of the present invention find use in directing the expression of DNA sequences encoding genes involved in a wide variety of plant genetic engineering applications. Such genes can encode for proteins involved in agronomic traits (input traits) such as herbicide tolerance and disease resistance, or quality traits (output traits) such as fatty acid composition modification and carotenoid production. Furthermore, DNA sequences encoding for proteins for the production of human biologics in a plant cell plastid also find use in the expression constructs of the present invention.

Constructs can also be prepared as to regulate the transcription and/or transcription and translation (expression) of a DNA sequence of interest from the plant cell plastid. Such

constructs are known in the art and are described in US Patent 5,576,198, the entirety of which is incorporated herein by reference. Such constructs can be used to direct the expression from cells of selected tissues in the host plant. For example, to direct the transcription and/or transcription and translation (expression) of a DNA sequence of interest from a plastid in a seed cell of a *Brassica* plant, promoters providing for enhanced expression in a seed are employed to direct the expression of T7 RNA polymerase from the plant cell nucleus.

Methods for the nuclear transformation of *Brassica* plants are known in the art, and are described for example in, Radke *et al.* (*Theor. Appl. Genet.* (1988) *75*:685-694 and *Plant Cell Reports* (1992) *11*:499-505).

Such methods can be used, for example, to modify the carotenoid biosynthetic pathway preferentially in seed plastids. Modification of carotenoid biosynthesis in seed is described for example in PCT Publication WO 98/06862, the entirety of which is incorporated herein by reference.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Example 1 Construction of Vectors

Constructs and methods for use in transforming the plastids of higher plants are described in Zoubenko *et al.* (*Nuc Acid Res* (1994) 22(19):3819-3824), Svab *et al.* (*Proc. Natl. Acad. Sci.*(1990) 87:8526-8530 and *Proc. Natl. Acad. Sci.*(1993) 90:913-917), Staub *et al.* (*EMBO J.* (1993) 12:601-606) and in U.S. Patent Number 5,576,198. The complete DNA sequences of the plastid genome of tobacco are reported by Shinozaki *et al.* (*EMBO J.* (1986) 5:2043-2049).

The T7 bacteriophage gene 10 leader (G10L) is constructed by ligating two oligonucleotides the potential stem-loop structure as well as the Shine-Delgarno sequence. The oligonucleotides used are 5'-AGGGAGACCACAACGGTTTCCCTCTAGAAA TAATTTTGTTTAACTTTAAGAAGGAGATATACC-3' (SEQ ID NO:1) and 5'-GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGAAACCGTTGTG GTCTCCCT-3'(SEQ ID NO:2).

A vector, pMON30125 was previously prepared to direct the integration and expression of a second mutated GFP (GFP-2)reporter gene and the aadA selectable marker gene from the plant plastid.

The GFP-2 was derived from the GFP-1 by two additional mutations (F64L and S65T, Cormack, *et al.*, (1996) *Gene* 173:33-38). Such mutations shift the excitation wavelength to blue light. The GFP-2 gene, also referred to herein as GFPuvm, was cloned between the Prrn/rbcL promoter/ribosome binding site and Trps16 transcription termination sequence. The P*rrn*/rbcL sequence is as described in Svab *et al.* (1993, *supra*). The T*rps*16 fragment comprises the *rps*16 gene 3'-regulatory region from nucleotides 5,087 to 4,939 in the tobacco plasmid DNA.

The expression cassette pMON30125 contains a marker gene, *aad*A, for selection on spectinomycin and streptomycin, and *rps* 7/12 for the integration, by homologous recombination, of the passenger DNA into *trn*V-*rps*7/12 intergenic region. The *aad*A marker gene is expressed from the *psb*A promoter and transcriptional termination sequences. The promoter region of the plastid psbA promoter (PpsbA) and terminator sequences (TpsbA) are described in Staub *et al.* (1993, *EMBO J.*, 12, 601-606).

In tobacco, homologous targeting of transgenes in the plastid genome between the *trnV* and *rps12/7* has yielded stable transformants at a high efficiency (Zoubenko et al. (1994) *supra*). Although the plastid genome of *Brassica* is collinear with that of tobacco, sequencing of this target region revealed several stretches of marked differences. Therefore, a *Brassica*-specific plastid transformation vector, pCGN6408, is constructed using approximately 5.0kb of target sequences derived from *Brassica*. PCGN6408 contains two transgene expression cassettes: 1) a selectable *aadA* gene conferring spectinomycin/streptomycin resistance and expressed using *Brassica* 16S*rrn* promoter region, bacterial T7 gene10 leader containing ribosome binding site which has been found to function efficiently in plastids (described in co-pending U.S. Patent Application 09/113,690) and *Brassica rps16* terminator sequences- *Prrn-g10L:aadA:Trps16*. 2) a green fluorescent protein (GFPuvm) marker gene expressed using *Brassica psbA* promoter and terminator sequences- *PpsbA:GFPuvm:TpsbA*. The two transgene expression cassettes are flanked by approximately 2.5kb plastid targeting sequences in pCGN6408.

The vector pCGN6408 is constructed by cloning the *GFPuvm* and *aadA* gene cassettes as an *Asc*I-*Not*I fragment from pCGN6399 into pCGN6407. pCGN6407 contains approximately 5.0 kb of plastid targeting sequences and is constructed by cloning

approximately 2.5kb flank sequence containing the *rps12/7* region as a *Not*I-*Sac*I fragment followed by cloning of *trnV* containing approximately 2.5kb flank sequence as an *Asp*718-*Xho*I fragment into pBluescript II KS (+) (Stratagene). The approximately 2.5kb flanks are PCR-amplified using *Brassica* total DNA, with primers (Table 1) SC175 and SC176 for *Asp*718-*Xho*I fragment and SC177 and SC178 for *Not*I-*Sac*I fragment. pCGN6399 is constructed by cloning the *Eco*RI-*Hind*III fragment containing the chimeric GFPuvm gene casette into pCGN6398, which contains the *aadA* expression cassette. The chimeric green fluorescent protein (GFPuvm) gene cassette, *PpsbA:GFPuvm:TpsbA*, has promoter and terminator sequences derived from Brassica *psbA*. It is constructed by cloning the GFPuvm expression cassette from pMON30125 into the *Eco*RI and *Hind*III sites of pUC118 and replacing the regulatory regions with the *PpsbA* as *Eco*RI-*Nco*I fragment and *TpsbA* as *Sac*I-*Hind*III fragment producing plasmid pCGN6388. The *PpsbA* is generated by PCR with primers SC186 and SC187 containing the appropriate restriction sites. Similarly, the *TpsbA* is PCR-amplified with primers SC188 and SC189 containing the appropriate restriction sites. The chimeric *aadA* gene has the *rrn* promoter (*Prrn*) fused with the gene-10 leader (*g10L*) from bacterial T7 phage and the terminator of *rps16* (*Trps16*). The *Trps16* is PCR-amplified primers SC193 and SC194 containing appropriate restriction sites, digested with *Sac*I and X*ba*I and cloned into pBluescriptII SK (+) (Stratagene) to generate pCGN6387. *Prrn* is serially PCR-amplified with primer pairs SC190 and SC191, SC190 and SC203, and SC190 and SC192 to generate a *Prrn-g10L* fusion. The *aadA* gene is PCR-amplified from pMON30125 with primers SC198 and SC199 . The Prrn-g10L is fused to aadA using overlapping primers SC198 and SC192, and the resulting Prrn-g10L:*aadA* fragment is amplified with primers SC190 and SC199. The Prrn-g10L:*aadA* fragment is digested with *Bam*HI and *Xba*I and cloned into pCGN6387. The resulting plasmid containing *Prrn-g10L:aadA:Trps16* is designated pCGN6398.

Sequences for the flanking regions as well as regulatory sequences for the marker and reporter genes are obtained from *Brassica napus* by PCR amplification with primers designed after known sequences of the same regions from other species. Conditions for the PCR reactions for amplifying the homology regions is as follows: 94°C for 4 min: 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min 45 s; with a final extension at 72°C for 7 min and for the regulatory regions: 94°C for 4 min: 30 cycles of 94°c for 15 s, 55°C for 30 s, 72°C for 30 s; with a final extension for 7 min. Primer sequences are listed in Table 1.

Table 1: Primer Sequences

NAME	SEQUENCE 5'-3'	SEQ ID
SC175	ACTGGGTACCCGAGTGAATAGAAAGTTGGATCTACATT G	SEQ ID NO:3
SC176	ACTGCTCGAGGCGCGCCGACAATTGAATCCAACTTTTT CCATTATTT	SEQ ID NO:4
SC177	ACTGGCGGCCGCAACTACTCCTATCGGAAATAGGATTG ACTA	SEQ ID NO:5
SC178	CATGGAGCTCGATCTCCCTCCAAACCGTACATACGACT	SEQ ID NO:6
SC186	GATCGAATTCATTTAATTAATTATATTTCTATGTATATA GATTCGTTTATAA	SEQ ID NO:7
SC187	GATCCCATGGTAAAATCCTTGGTTTATTTAATCATC	SEQ ID NO:8
SC188	GATCGAGCTCTACAAATAATGATCTAGATTCTTTAGTG TTAGTCTATACCTAGT	SEQ ID NO:9
SC189	GATCAAGCTTAGGCGGCCGCACGCAGCAATATTTTTTT TGATAA	SEQ ID NO:10
SC190	GATCGGATCCAAGGCCGGCCGACTTGCTCCCTCGCTGT GATCGAATAAG	SEQ ID NO:11
SC191	TTTCTAGTGGGAAACCGTTGTGGTCTCCCTACAAAGCT GATTCGGAATTGTCTTTC	SEQ ID NO:12
SC192	CGATACTTCGGCGATCACCGCTTCCCTTCCCATGGGTA TATCTCCTTCTTAAAGTT	SEQ ID NO:13
SC193	GATCTTCTAGAACTATAAAAAAGAGGATGTTAAAGACT CATATATAGCTTG	SEQ ID NO:14
SC194	GATCGAGCTCATGGCGCGCCATTTTATATATTTTCTATA CAATAATTCTATAC	SEQ ID NO:15

NAME	SEQUENCE 5'-3'	SEQ ID
SC198	AGAAGGAGATATACCCATGGGAAGGGAAGCGGTGATC GC	SEQ ID NO:16
SC199	ACGTTCTAGAATTATTTGCCGACTACCTTAGTGATCTCG	SEQ ID NO:17
SC203	TATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGT GGGAAACCGTTGTG	SEQ ID NO:18

A transformation vector is prepared to direct the integration and expression of a reporter and marker genes from the plant plastid using the flanking regions and the regulatory regions obtained above.

The vector pCGN6408 was constructed by cloning the *GFPuvm* and *aadA* gene cassettes as an *Asc*I-*Not*I fragment from pCGN6399 (described below) into pCGN6407(described below). The plasmid pCGN6407 contains approximately 5.0 kb of *Brassica* plastid targeting sequences and was constructed by cloning approximately 2.5kb flanking sequence containing the *rps12/7* region as a *Not*I-*Sac*I fragment followed by cloning of *trnV* containing approximately 2.5kb flank sequence as an *Asp*718-*Xho*I fragment into pBluescript II KS (+) (Stratagene). The approximately 2.5kb flanks were PCR-amplified using *Brassica* total DNA, with primers SC175 and SC176 for *Asp*718-*Xho*I fragment and SC177 and SC178 for *Not*I-*Sac*I fragment. pCGN6399 was constructed by cloning the *Eco*RI-*Hind*III fragment containing the chimeric GFPuvm gene casette into pCGN6398, which contains the *aadA* expression cassette. The chimeric green fluorescent protein (GFPuvm) gene cassette, *PpsbA:GFPuvm:TpsbA*, has promoter and terminator sequences derived from Brassica *psbA*. It was constructed by cloning the GFPuvm expression cassette from pMON30125 into the *Eco*RI and *Hind*III sites of pUC118 and replacing the regulatory regions with the *PpsbA* as *Eco*RI-*Nco*I fragment and *TpsbA* as *Sac*I-*Hind*III fragment producing plasmid pCGN6388. The *PpsbA* was generated by PCR with primers SC186 and SC187 containing the appropriate restriction sites. Similarly, the *TpsbA* was PCR-amplified with primers SC188 and SC189 containing the appropriate restriction sites. The chimeric *aadA* gene has the *rrn* promoter (*Prrn*) fused with the gene-10 leader (*g10L*) from bacterial T7 phage and the terminator of *rps16* (*Trps16*). The *Trps16* was PCR-amplified primers

SC193 and SC194 containing appropriate restriction sites, digested with *Sac*I and X*ba*I and cloned into pBluescriptII SK (+) (Stratagene) to generate pCGN6387. *Prrn* was serially PCR-amplified with primer pairs SC190 and SC191, SC190 and SC203, and SC190 and SC192 to generate a *Prrn-g10L* fusion. The *aadA* gene was PCR-amplified from pMON30125 with primers SC198 and SC199 . The Prrn-g10L was fused to aadA using overlapping primers SC192 and SC198 and the Prrn-g10L:aadA fragment amplified by primers SC190 and SC199. The Prrn-g10L:*aadA* fragment was then digested with *Bam*HI and *Xba*I and cloned into pCGN6387. The resulting plasmid containing *Prrn-g10L:aadA:Trps16* was designated pCGN6398.

The nucleic acid sequence of the *Brassica* plastid expression cassette is determined to confirm the sequences of the homology regions and the regulatory sequences, and is provided in SEQ ID NO:19.

Example 2 Chloroplast Transformation

A. Establishment of Source Plants

Seeds of *Brassica napus* variety 212/86 are surface sterilized by immersion in 95% ethanol for 2 minutes, followed by 30 minutes in 20% Clorox with 1 drop of dish soap per 100ml as a surfactant. The seeds are rinsed four times in sterile distilled water.

The sterilized seeds are sown in Magenta boxes (Magenta Corp. Chicago, IL) containing Brassica germination medium (Table 2). The boxes are placed in a growth room at 21°C with a 16hr light period and a light intensity of 55 photons/meter^2/sceond. Cotyledons are removed from two week old plants and the upper part of the shoots are excised from the roots and placed on *Brassica in vitro* plant medium (Table 3), one plant per box. Plants are grown under the same conditions as seedlings and are subcultured using the *in vitro* medium every four weeks.

Table 2: Germination Medium

Component	Concentration
Murashige Minimal Organics	3.457g/L
Pyridoxine-HCL	0.05mg/L
Nicotinic Acid	0.05mg/L
Glycine	0.2mg/L
Phytagar	6g/L
pH adjusted to 5.8	

Table 3: In Vitro Plant (IVP) Medium

Component	Concentration
10x B5 Salts (Table 4)	1x
100x B5 Vitamins + inositol (Table 5)	1x
Sucrose	30g/L
Phytagar	6g/L
pH adjusted to 5.8	

Table 4: 10x B5 Salts

Component	Concentration
Ammonium Sulfate	134 mg/L
Boric Acid	3 mg/L
Calcium Chloride $\bullet 2H_2O$	150 mg/L
Cobalt Chloride $\bullet 6H_2O$	0.025 mg/L
Cupric Sulfate $\bullet 5H_2O$	0.025 mg/L
EDTA (Disodium Salt) $\bullet 2H_2O$	37.3 mg/L
Ferrous Sulfate $\bullet 7H_2O$	27.8 mg/L
Magnesium Sulfate $\bullet 7H_2O$	250 mg/L
Manganese Sulfate $\bullet H_2O$	10 mg/L
Molybdic Acid (Sodium Salt) $\bullet 2H_2O$	0.25 mg/L
Potassium Iodide	0.75 mg/L
Potassium Nitrate	2500 mg/L
Sodium Phosphate Monobasic $\bullet H_2O$	150 mg/L
Zinc Sulfate $\bullet 7H_2O$	2 mg/L

Table 5:100x B5 Vitamins + Inositol

Component	Concentration
Inositol	10 g/L
Nicotinic Acid	0.1 g/L
Pyridoxine-HCL	0.1 g/L
Thiamine-HCL	1 g/L
pH adjusted to 5.6	

5

B. Bombardment Conditions

Tungsten or gold particles are sterilized for use as microcarriers in bombardment experiments. Gold particles ((1.4mg, enough for 5 bombardments) 0.6 micron gold particles

(#1652262. Bio-Rad Laboratories) are sterilized with 100% ethanol. Immediately prior to use, particles are sedimented by centrifugation, washed with 2 to 3 washes of sterile deionised distilled water, vortexed and centrifuged between each wash. Washed particles are resuspended in 50µl of sterile water.

To the 50ul of sterile gold particles is added 10µl of a 1ug/µl solution containing pCGN6408 DNA in TE buffer. Also added was 50µl of 2.5M CaCl2 and 20 µl of 0.1M spermidine free base. The particles are shaken for 10 minutes at 4°C, centrifuged washed 4 times in 100% ethanol. The final suspension in absolute ethanol is 30µl. Five microliters of this suspension is loaded on a flying disc for use in particle bombardments.

Dark green leaves, about ¾ expanded, approximately 3 to 4 cm in length, are chosen for bombardment from the source plants. Leaves are cut, leaving no petiole and placed with the abaxial side against the Shooting medium (Table 6) in petri dishes. Immediately before shooting leaves are gently pressed flat in contact with the medium.

Table 6: Shooting Medium

Component	Concentration
10x B5 Salts (Table 4)	1x
100x B5 Vitamins + Inositol (Table 5)	1x
Sucrose	30 g/L
BAP	1 mg/L
Phytagar	6 g/L
pH adjusted to 5.8	

Transformation by particle bombardment is carried out using the PDS 1000 Helium gun (Bio Rad, Richmond, CA) using a modified protocol described by the manufacturer.

The flying disc was placed in a launch ring, which is screwed into a sleeve with a metal stopping screen on a retainer ring 1 cm below the launch ring. The flying disc had an effective flight distance of 1 cm. Leaves are placed on the second platform from the bottom of the gun, 12 cm from the stopping screen and bombarded using a 1100 p.s.i. rupture disk.

C. Regeneration of Transformed Shoot

After bombardment, leaves are left on the shooting medium plates and are sealed with ¼" Micropore tape (3M Health Care, St. Paul, MN 555144) and placed in the culture room at 21°C for two days with light conditions of 90 photons/meter^2/sec and a photoperiod of 16 hours light. After two days, the leaves are removed from the media and cut into approximately 1 cm squares. The squares are placed abaxial side against Brassica Regeneration medium (Table 7) supplemented with 20 mg/l spectinomycin. After seven days on Brassica Regeneration medium leaf pieces are moved to Shoot development medium (Table 8) supplemented with 20mg/l spectinomycin, abaxial side against the medium. After two weeks,the leaf pieces are moved to fresh Shoot development medium containing 20mg/l spectinomycin.

Both bleached (purple/white) shoots and green shoots regenerated about 6 to 8 weeks after bombardment. Green shoots, approximately 0.25 to 0.5 cm in height, and a small part of the explant are moved to fresh Shoot development medium with 20mg/l spectinomycin. A green shoot (99-A), elongated to approximately 1 cm in height and was removed from the explant and placed on shoot elongation medium (Table 9) plus 20mg/l spectinomycin. At 2 cm in height the shoot is placed on rooting medium supplemented with 20 mg/l spectinomycin.

Table 7: Regeneration Medium

Component	Concentration
10x B5 Salts (Table 4)	1x
100x B5 Vitamins + Inositol (Table 5)	1x
$AgNO_3$	3 mg/L
Sucrose	30 g/L
N6-benzylaminopurine(BAP)	5 mg/L
Naphthaleneacetic acid (NAA)	5 mg/L
Phytagar	6 g/L
pH adjusted to 5.8	

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Table 8: Shoot Development Medium

Component	Concentration
10x B5 Salts (Table 4)	1x
100x B5 Vitamins + Inositol (Table 5)	1x
BAP	3 mg/L
Zeatin	1 mg/L
Sucrose	10 g/L
Phytagar	7 g/L
pH adjusted to 5.8	

Table 9: Shoot Elongation Medium

Component	Concentration
10x B5 Salts (Table 4)	1x
100x B5 Vitamins + Inositol (Table 5)	1x
Sucrose	10 g/L
Phytagar	6 g/L
pH adjusted to 5.8	

Table 10: Rooting Medium

Component	Concentration
10x B5 Salts (Table 4)	1x
100x B5 Vitamins + Inositol (Table 5)	1x
Sucrose	10 g/L
IBA	1 mg/L
Phytagar	6 g/L
pH adjusted to 5.8	

D. Regeneration of Homoplasmic Shoots

Leaves are removed from the heteroplasmic shoot from shot 99, cut into 0.5 cm pieces and placed abaxial side against the medium on Regeneration medium supplemented with 20 mg/l spectinomycin. After seven days on Brassica Regeneration medium leaf pieces are moved to Shoot development medium with 20mg/l Spectinomycin, abaxial side against the medium. Two weeks later the leaf pieces were moved to fresh to Shoot development medium plus 20mg/l spectinomycin. Green shoots regenerated about 2 to 3 weeks after leaves were cut up. Green shoots and a small part of the explant were moved to fresh to Shoot development medium plus 20mg/l spectinomycin at approximately 0.25 to 0.5 cm in height. At approximately 1 cm in height, shoots were cut off the explant and transferred to shoot

elongation medium plus 20mg/l spectinomycin. At 2 cm in height shoots were placed on rooting medium (table 10) containing 20 mg/l spectinomycin.

Example 3 Analysis of Transplastomic Plants

Transformed plants selected for marker *aad*A marker gene expression are analyzed to determine whether the entire plastid content of the plant has been transformed (homoplastic transformants). Typically, following two rounds of shoot formation and spectinomycin selection, approximately 50% of the transgenic plantlets which are analyzed are homoplastic, as determined by Southern blot analysis of plastid DNA. Heteroplasmic plantlets are selected for further subculturing to obtain homoplasmic lines.

Genomic DNA can be isolated from transformed *Brassica* plants, electrophoresed, and transferred to filters as generally described by Svab *et al.* ((1993), *Proc Natl Acad Sci*, 90:913-917). Plastid transformants are identified by Southern analysis after digestion of total DNA with *StuI* restriction enzyme.

Total DNA is extracted from leaves using plant DNAzol Reagent (GIBCO BRL), digested with *StuI*, electrophoresed on 0.7% agarose gels and transferred to nylon membranes (Amersham) using standard techniques. Blots are probed using Rapid Hybridization buffer (Amersham) using ^{32}P-labeled PCR amplified DNA probe obtained with primers SC177 and SC178 from pCGN6408. The probed region comprises the entire *Not*I-*Sac*I flank sequence of *Brassica* plastid vector pCGN6408 (Figure 1). This probe detects a 4.50 kb fragment in wild type Brassica napus 212/86 DNA and a 7.02 kb transgenic fragment in Bn-pCGN6408-1 line and its secondary regenerates Bn-pCGN6408-1-1, Bn-pCGN6408-1-2, Bn-pCGN6408-1-3 and Bn-pCGN6408-1-4. The presence of both wild-type size and transgenic fragments in the transgenic lines indicates that they are heteroplasmic for the transformed plastid DNA (Figure 2).

Homoplasmic plants are obtained through further subculturing using excised leaf tissue for regeneration of plants using the regeneration methods described in Example 2C. Homoplasmic *Brassica* plants are identified using Southern blot hybridizations as described above and selecting lines demonstrating a single hybridizing fragment of 7.02 kb corresponding to the transplastome. Figure 3 provides Southern Hybridization results for subsequent lines. Lines 6408 99-1Y, cc, and dd are about 80% homoplasmic.

Example 4 Analysis of GFP Expression

Leaves from heteroplasmic shoot (Bn-6408-99-1) and an nontransformed control shoot are removed and sliced tangentially. Prepared leaves are observed with a Nikon Labophot fluorescent microscopc and photos are taken with a Nikon UFX-II photomicrography unit.

Visual observation of the prepared tissues revcals fluorescence from the chloroplasts in the guard cells of stomates under blue light. Thus, demonstrating GFP expression from the *Brassica* plant cell plastid.

Quantitative analysis of GFP expression can be determined using Western Immunoblot analysis. For example, total soluble protein can be extracted from frozen or frozen leaf tissue by grinding 250 mg tissue in 250µl of PBS buffer (1 mM KH_2PO_4, Na_2HPO_4, 0.137M NaCl, 2.7 mM KCl pH 7.0) containing protease inhibitors. The homogenate is centrifuged for 5 minutes, and the supernatant is transferred to a fresh tube. The concentration of the protein in the supernatant is determined using a protein concentration assay (BioRad, Richmond, CA).

Extracted total protein is electrophoresed on a 4-20% SDS-PAGE gel (Sigma, St Louis, MO), and transferred to PVDF membrane in 1x SDS-PAGE buffer (Maniatis *et al.* 1989, Cold Spring Harbor Press). Standards of quantitated purified GFP protein are used to quantify the expression of the GFP as expressed in the plant plastid.

Western hybridizations are performed as described in Staub and Maliga (1993) *EMBO Journal*, 12(2) 601-606, except using antibodies raised to GFP. PVDF membranes containing the transferred electrophoresed protein are incubated in a blocking solution of PBS buffer containing 0.05% Tween-20 (PBS-T) and 5% milk overnight at 4°C. The membranes are then incubated in a solution of PBS-T containing 1% milk and a primary antibody raised in goats to GFP for 2 hours at room temperature. The membranes are washed three times in a solution of PBS-T containing 0.1% milk, each wash for 5 minutes at room temperature. The membranes are then incubated in a solution of PBS-T containing 1% milk and sheep anti-goat antibody for 1 hour at room temperature, and washed again in PBS-T containing 0.1% milk, three times for 10 minutes at room temperature. A final wash using only PBS-T is performed before developing the membranes using, for example, a nonradioactive detection kit (ECL, Amersham).

Thus, the above results demonstrate that the methods provided in the present invention allow for the transformation of *Brassica* plant cell plastids.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications can be practiced within the scope of the appended claim.

Claims

What is claimed is:

1. A method for the transformation of a *Brassica* plant cell comprising: introducing into plastids of said cell a construct having a promoter functional in a plant plastid operably associated with a DNA sequence of interest.

2. The method according to Claim 1, further comprising selecting cells having plastids containing said construct.

3. The method according to Claim 1, wherein said construct comprises regions of homology to the genome of said plastid whereby said construct is integrated into the plant cell plastid genome.

4. The method according to Claim 3, wherein said regions of homology are derived from a *Brassica* plastid genome.

5. The method according to Claim 2 further comprising regenerating a mature plant containing transformed plastids from said plant cells.

6. The method according to Claim 1 wherein said plant cell is a *Brassica napus* cell.

7. The method according to Claim 1 wherein said plant cell comprises a leaf cell.

8. The method according to Claim 1, wherein said DNA sequence of interest is a DNA sequence capable of conferring tolerance in a plant cell to at least one herbicide compound when said DNA sequence is transcribed in plastids of said plant cell.

9. The method according to Claim 1, wherein said DNA sequence of interest encodes a gene from *Bacillus thuringensis* providing for resistance to insects.

10. The method according to Claim 1, wherein said DNA sequence of interest encodes a gene capable of directing the production of a human biological protein.

11. A plant cell obtained by the method of Claim 1.

12. A plant, plant seed or plant part or progeny thereof containing a plant cell according to Claim 10.

13. A *Brassica* plant cell having plastids containing a heterologous construct, wherein said construct comprises, as operably associated components in the 5' to 3' direction of transcription, a promoter functional in a plant cell plastid, a DNA sequence of interest and a transcriptional terminator functional in a plant cell plastid.

14. A plant cell according to Claim 13, wherein said construct further comprises regions of homology to the genome of said plastid whereby said construct is integrated into the plant cell plastid genome.

15. The plant cell according to Claim 13, wherein said regions of homology are derived from a *Brassica* plastid genome.

16. The plant cell according to Claim 13 wherein said plant cell is a *Brassica napus* cell.

17. The plant cell according to Claim 13 wherein said plant cell comprises a leaf cell.

18. A plant, plant seed or plant part or progeny thereof containing a plant cell according to Claim 13.

19. A recombinant nucleic acid construct comprising: a promoter functional in a plant cell plastid; a nucleic acid sequence of interest, and regions of homology derived from a *Brassica* plastid genome.

20. The construct according to Claim 19, wherein said regions of homology are derived from *Brassica napus*.

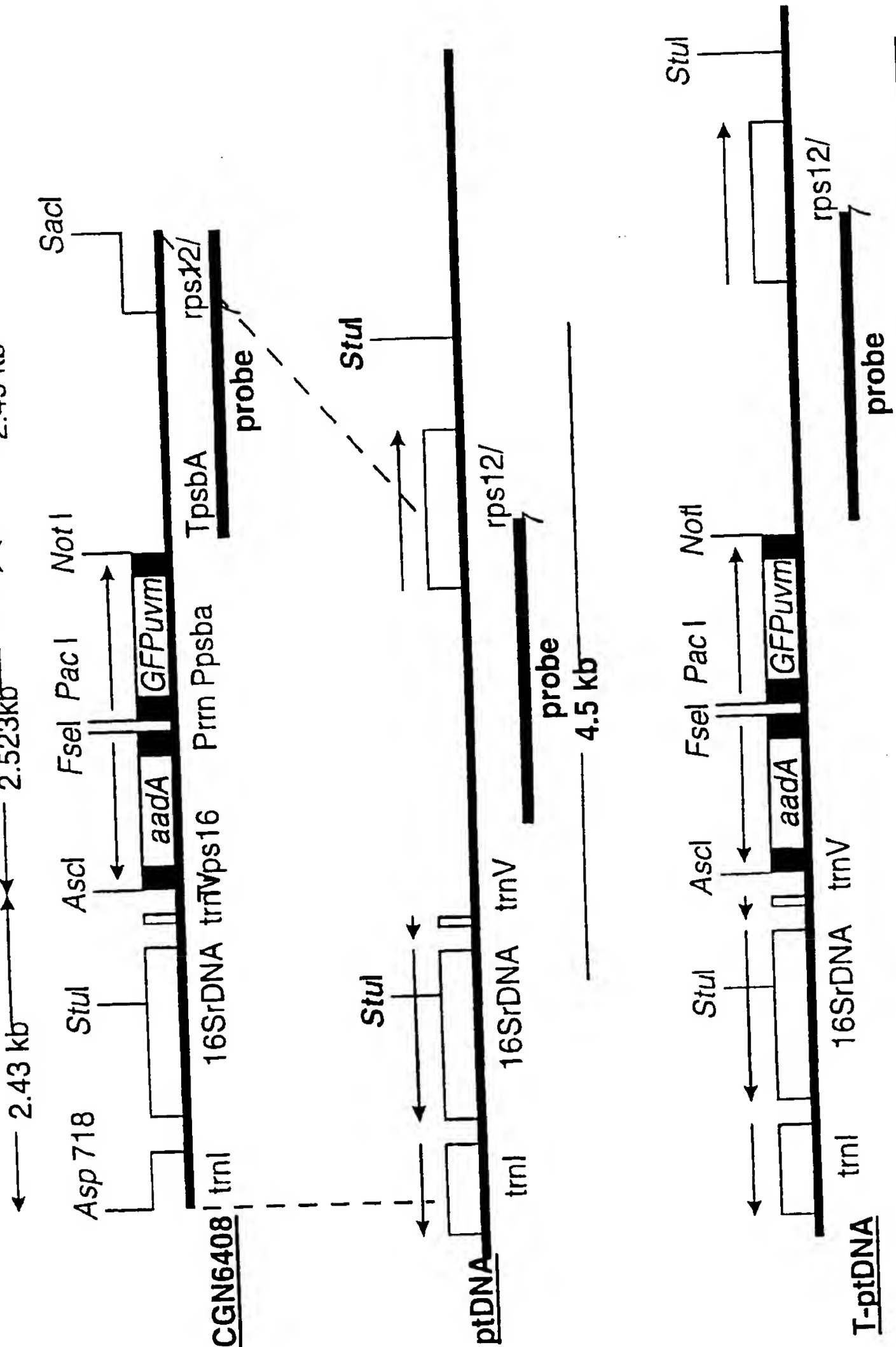
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Figure 1

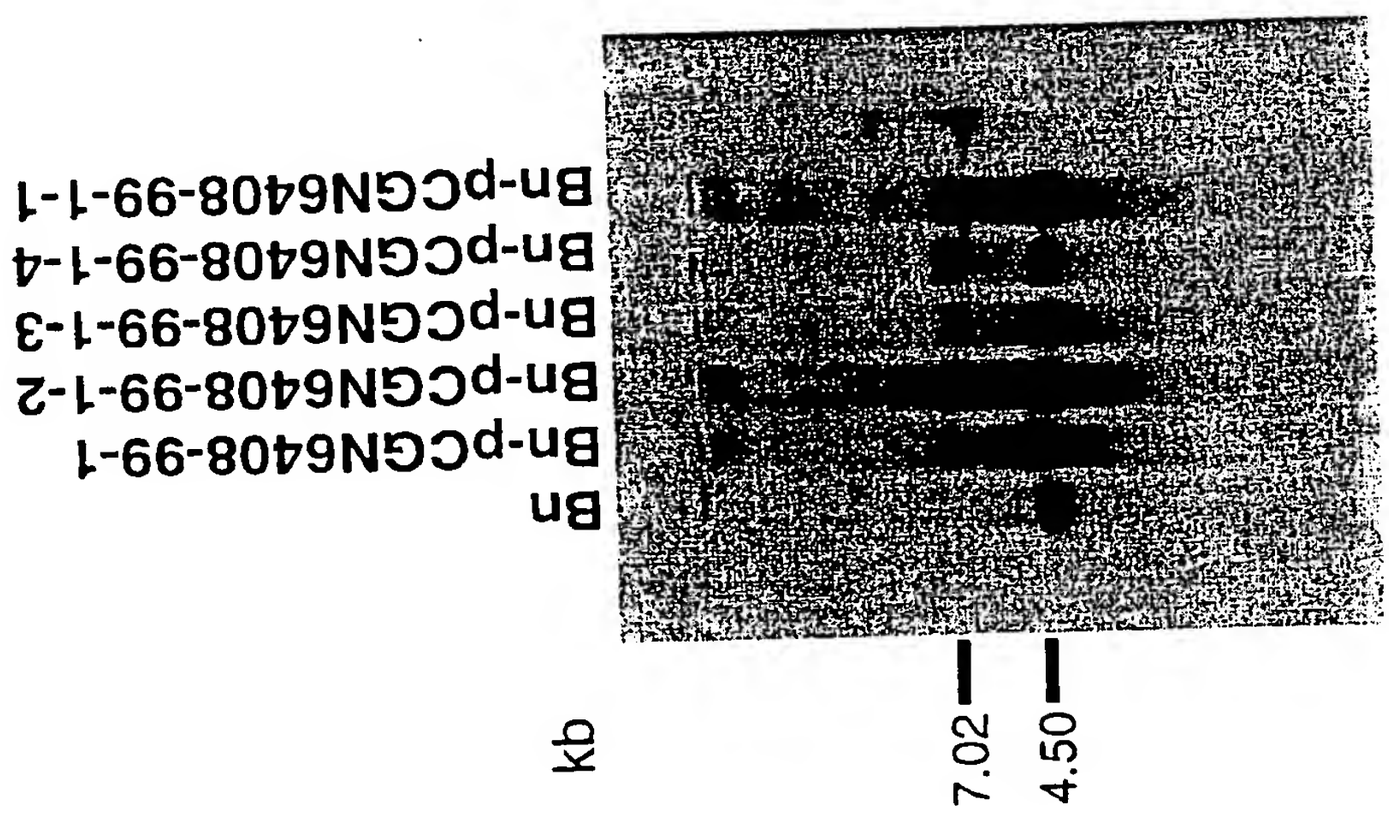
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Figure 2

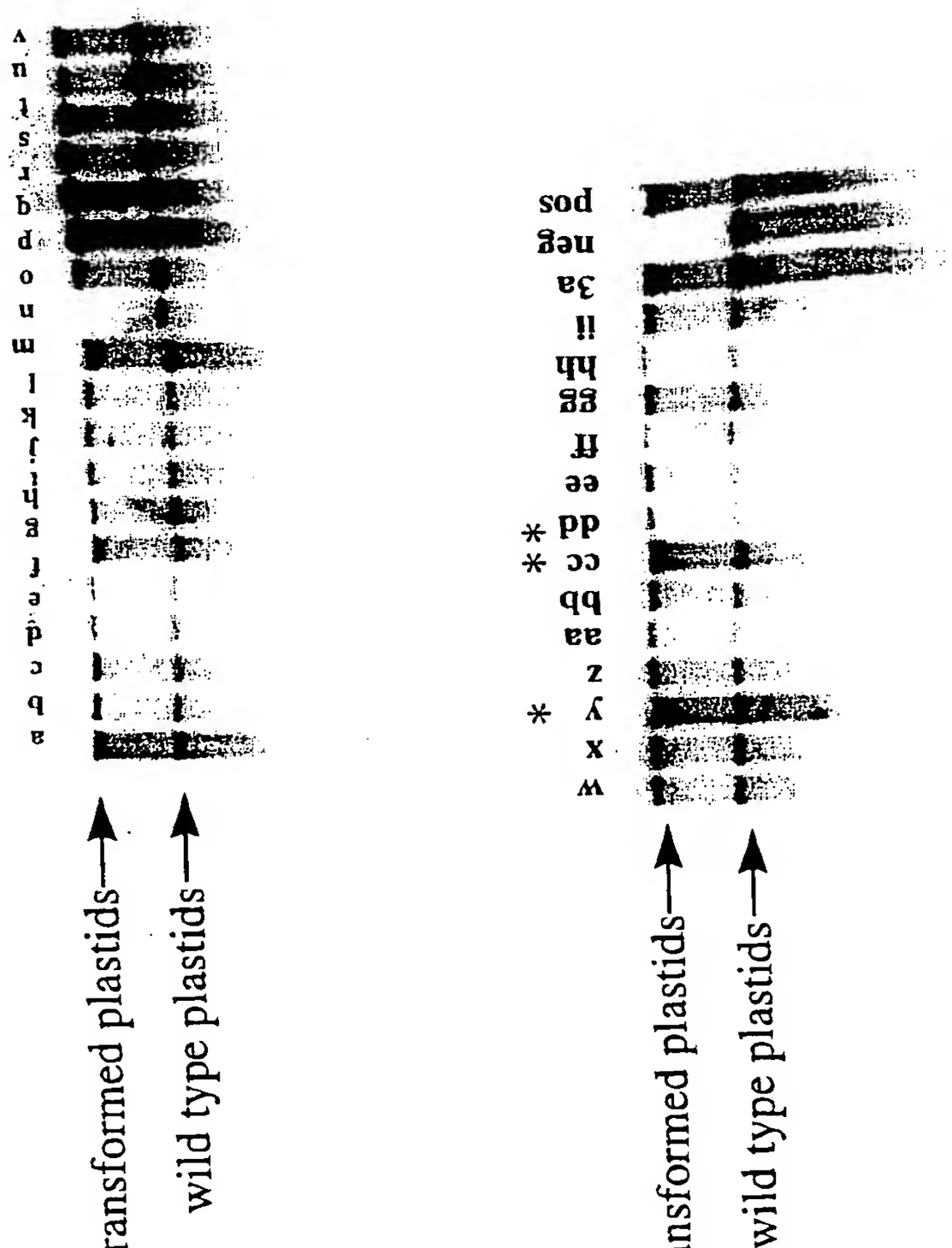
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Figure 3

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/30183

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROESLER, KEITH ET AL: "Targeting of the Arabidopsis homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds." PLANT PHYSIOLOGY (ROCKVILLE), (1997) VOL. 113, NO. 1, PP. 75-81. , XP000891347 the whole document ---	1-6, 11-20
X	WO 97 32977 A (UNIV RUTGERS ;MALIGA PAL (US); SIKDAR SAMIR R (IN); REDDY SIVA VAN) 12 September 1997 (1997-09-12) cited in the application example 3 ---	1-7, 11-20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2000	02/05/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Hix, R

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/30183

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Inte onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	ZORA SVAB ET AL: "STABLE TRANSFORMATION OF PLASTIDS IN HIGHER PLANTS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 87, no. 21, 1 November 1990 (1990-11-01), pages 8526-8530, XP000164753 ISSN: 0027-8424 cited in the application the whole document ---	
A	P. MALIGA: "Towards plastid transformation in flowering plants." TRENDS IN BIOTECHNOLOGY, vol. 11, no. 3, March 1993 (1993-03), pages 101-107, XP000892855 cited in the application the whole document -----	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 99/30183

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